# Threshold Effect of Free Radical Quenching in a Progressive Breast Cancer Cell line Model

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***Abstract***

Oxidative stress is a consequence of both normal and abnormal cellular metabolism and is linked to cell proliferation, differentiation and apoptosis leading to the development of human diseases. A common mechanism for chemotherapeutic agents inducing cell death is through the generation of free radicals. Although the exact mechanism of the molecular signalling that it entails is still being worked upon, it is clear that this varies with the stage and type of cancer and the drug and dosage used. We study the response of MCF10A series progressive breast cancer cell lines, in response to Adriamycin and Cyclophosphamide. A targeted set of candidate genes from these pathways that participate in free radical metabolism were evaluated for gene expression. Genes in oxidative stress response pathways were modelled earlier using the multivariate Boolean Network Modelling. We provide evidence that the strategy of using Boolean modelling and laboratory testing of the model, although not a perfect match, has potential to contribute in biology. We present a novel and testable "threshold" model of free radical generation that is dependent on the quantity of free radical generation. Using logic tools like Boolean circuitry, with fine-tuned experiments may serve a useful purpose in designing preclinical chemotherapic protocols.his document gives formatting instructions for authors preparing papers for publication in this journal. All authors must follow the instructions given in this document for the papers to be published.

***Keywords***

*Breast Cancer; Boolean Model; Chemotherapy; Bioinformatics*

## Introduction

Oxidative free radicals are continuously generated in the human body as a direct consequence of aerobic metabolism [1] . The outcome of interactions between the cell and oxidants is largely determined by the balance between the free radicals and the cellular antioxidants and enzymes that detoxify these reactive intermediaries. Oxidative stress results when the balance tilts towards excess free radicals. These free radicals are also capable of causing mutations in the DNA and, if the stress persists over extended periods of time, can lead to excessive cellular proliferation and cancer. Oxidative stress is also known to contribute to aging, cardiovascular disease, chronic inflammation and neurodegenerative diseases. However, in certain cases excessive free radical generation in a cell can also cause it to undergo programmed cell death or apoptosis. This two faced character of free radicals excessive cell proliferation or cell death is a key reason why the research community is still debating whether an excess of free radicals should be averted or utilized as an effective therapeutic device against cancer cells. On the other hand, there is still an enthusiastic on-going debate as to whether the activation of antioxidant defences would improve cancer treatment or, to the contrary help protect initiated cells against oxidative toxicity and apoptosis [2], [3]. Added to this continuing debate several chemotherapeutic drugs have been found to work by inducing free radicals and hence oxidative stress has been suggested as the mechanism that kills the cells. However, there are recent alternative theories that suggest the desired results obtained with these drugs are not necessarily achieved through the production of free radicals, but may represent merely a consequence of other mechanisms of drug action [4].

Breast cancer is the most frequently diagnosed cancer in women in the United States, with an estimated 232,340 new cases of invasive disease in 2013 as per U.S Breast Cancer Statistics. Despite a decrease in breast cancer


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incidence rates in the United States, it remains the second leading cause of cancer deaths in American women, frequently due to metastasis to different organ systems [5]. Although we have made progress, our current understanding of the biology of breast cancer coupled with identification of optimal therapy is necessary to make a dent in the progression of this disease. To this end breast cancer cell lines have been the most widely used models to investigate the molecular mechanism driving breast cancer progression including cancer cell proliferation, migration and apoptosis[6]. The use of cell lines has resulted in a wealth of information about the genes and signaling pathways that regulate these processes. Human breast cancer cell lines have served as the principal models for breast cancer research since they are easily propagated, amenable to genetic manipulation and, under welldefined experimental conditions, yield reproducible and quantifiable results. Most importantly breast cancer cell lines share many of the genetic and genomic features of human breast cancers, including representing several breast cancer subtypes. Several breast cancer cell lines serve as models to investigate both tumour-initiation and tumour cell progression cell properties. [7]. The four MCF10A series of cell lines employed in this study represent breast cancer progression from near normal epithelial cells towards overt malignancy and have proven to be a powerful model to study breast cancer progression [8], [9]. Although in recent years there have been apparent advances and developments in understanding how free radical generation contributes to both progression and prognosis of breast cancer there still remains several basic unanswered questions. Especially free radical generation as it relates to chemotherapy and tumour cell behaviour based on its progression from normal to its acquiring metastatic potential. As against this background, the primary purpose of this study is to understand how the progressive model of breast cancer MCF-10A cell lines, responds to drug treatment both through free radical generation and through gene expression alterations of key genes in the free radical pathway. The second rationale for this work was to test the validity of a generalized Boolean (multivariate) model of ROS signaling we proposed [10]. Furthermore, our intent was not only to generate experimental evidence to validate the model but seek further insights in ROS signalling networks that will help design future experiments and models through a iteration process. Such approaches where in model and experimental testing are executed in parallel, within the paradigm of systems biology, the new frontier [11], [12]. The drugs tested were Adriamycin and Cyclophosphamide two commonly used drugs for the treatment of breast cancer the most frequently used therapeutic dosage was selected for this study.

## Materials and Methods

### *Functional Cell Line Grouping*

The cell lines used, namely MCF10A, MCF10AneoT, MCF10CA1h and MCF10CA1a [13], [14]are a series of cell lines that provide a distinct opportunity to understand the progressively malignant nature of breast cancer. MCF10A cell line is spontaneously immortalized human mammary epithelial cells with near diploid karyotype harboring a number of chromosome abnormalities. Besides being frequently used in in vitro transformation assays MCF10A cell line was used for comprehensive analysis of the MCF10A series of cell lines representing progression towards recognizable malignancy [8], [9]. The MCF10A progression model consists of three directly derived cell lines: the spontaneously immortalized MCF10A cells (do not show any characteristics of invasiveness or tumor formation), MCF10AT1 cells (MCF10A cells transformed by HRAS), and MCF10CA1a cells (obtained from MCF10AT1 cells after xenograft transplantation in immune-deficient mice[8], [9]. Each of these cell lines showed specific quantitative chromosomal changes in addition to carrying a few common changes seen in all cell lines [13], [14]. Spectral karyotyping analysis showed that qualitative changes for example premalignant MCF10AT1 gained additional translocations to the MCF10A, whereas the malignant MCF10CA1a had more translocations than both MCF10A and MCF10AT1[8]. Array comparative genome hybridization (aCGH) showed that MCF10A had a number of gains and losses of different chromosome regions and progression towards full malignancy was accompanied by much more widespread genomic aberrations. Importantly, regions of genomic loss/gain overlapped only partially among these three cell lines [13], [14]. MCF10A series of cell lines has been also used for confirming the stepwise genome changes accompanying progression to full malignancy[9]. Furthermore, combining SNP array with Gene Array authors showed correlation between DNA copy number gains and increased expression levels for genes located in these regions[9].The induction of this progression to carcinogenesis in a molecularly defined way with a shared cell background makes it a unique model which can be exploited for

functional studies, especially as it relates to altered functions and concomitant genetic changes. Based on this functional capacity, the cell lines were grouped distinctly into two groups for our analysis purposes: the non-tumorigenic MCF10A and MCF10AneoT group and the tumorigenic MCF10CA1h and MCF10CA1a group.

***Cell lines, Chemo-Drug Exposure, Total RNA Extraction and cDNA Synthesis***

MCF-10A, MCF-10AneoT, MCF-10CA-1h and MCF-10CA-1a cells were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI). The four cell lines were maintained in monolayer in Dulbecco's modified Eagles medium-F12 (DMEM/F12) (Invitrogen, USA), supplemented with 5% horse serum (Invitrogen), 1% pencillin/streptomycin (Invitrogen), 0.5ug/ml hydrocortisone, 100ng/ml cholera toxin (Sigma,USA) 10ug/ml insulin and 20ng/ml recombinant human EGF (preprotech, USA). All cell cultures were incubated at 370 C in a 5% CO2 incubator. Cyclophosphamide for Injection 500mg/ml USP (Baxter, USA) and Doxorubicin HCL for injection USP 2mg/ml were purchased from APP pharmaceuticals (USA). Concentrations used were derived from the usual chemotherapeutic dose of (60 mg/m2 and 600 mg/m2) respectively using plain(DMEM/F12) medium without serum. Total RNA was extracted after 4 hours and 24 hours exposures. Total RNA was extracted by using Aurum total RNA mini spin column (Biorad, USA), treated with DNAse I and recovered exactly per the manufacturers' instruction. DNA free total RNA was quantified (Nanodrop ND-1000 spectrophotometer; Thermo Scientific). 1μg of each RNA was converted to the first strand cDNA using iScript reverse transcription supermix (Biorad) for RT-qPCR as per the manufacturer's instruction. Both RNA and cDNA were evaluated for quality through ND-1000 spectrophotometer based 260/280 OD ratio and 1% agarose gel for 28S/18S visualization.

The qPCR reaction was carried out in 10-μL volumes using the 2×iQ SYBR Green Supermix (Bio- Rad) per kit instructions with 50ng of cDNA, 500 nM of forward and reverse primers. The qPCR reactions were performed on a CFX384 Touch™ Real-Time PCR Detection System(Bio-Rad) using the following cycling conditions 95°C for 3 minutes followed by 35 cycles at 95°C for 15 seconds,60°C for 20 seconds, and 72°C for 20 seconds. Melt-curve analysis was carried out to ensure specific primer binding. mRNA abundance was calculated using the -2 (ΔΔ C(T)) method as previously describe[15]. Expression of each transcript was normalized to HGPRT as the house keeping gene and displayed as -fold change. Each sample was analyzed in triplicates. Integrated software with CFX384 Real-Time PCR Detection System -Biorad CFX384 manager (V3.0) was used to calculate the significance of gene expression differences ($P < .05$ is deemed significant).

***Sequences of Primers Used for RT-qPCR Validation of ROS Regulatory Genes***

Gene Name qPCR Sequence

BACH1-F TGT TGT CGG GAA GTT CAG TG
BACH1-R GCT CTC GCT TCA GTC AGT CG
NRF2-F GCT CAT ACT CTT TCC GTC GC
NRF2-R GAC TCC CGT CCC AGC AG
KEAP1-F GCT GAT GAG GGT CAC CAG TT
KEAP1-R CCA ACT TCG CTG AGC AGA TT
NQO1-F GCA TAG AGG TCC GAC TCC AC
NQO1-R GGA CTG CAC CAG AGC CAT
HMOX1-F GGC ATA AAG CCC TAC AGC AA
HMOX1-R GCC AGC AAC AAA GTG CAA G
PKC-F CAA ATT CAT GGC ACC TCT TG
PKC-R CAC TGC ACC GAC TTC ATC TG
SOD1 F CCA CAC CTT CAC TGG TCC AT
SOD1 R CTA GCG AGT TAT GGC GAC G
SOD2 F TAG GGC TGA GGT TTG TCC AG
SOD2 R GGA GAA GTA CCA GGA GGC GT
SOD3 F CGA GTC AGA GTT GGG CTC C
SOD3 R TCT CTT GGA GGA GCT GGA AA

### *Detection of Reactive Oxygen Species*

Samples were prepared in duplicate and incubated for 30 min with CM-H2DCFDA, a fluorescence probe sensitive to cellular oxidants such as hydrogen peroxide (H2O2), hydroxyl radicals (OH), and peroxyl radicals (OOH). This probe passively diffuses into cells and upon oxidation by ROS forms a fluorescent adduct which remains trapped in the cell. Fluorescence images were collected, as previously described by us [16], with a Stallion Digital Imaging workstation (Carl Zeiss) equipped with a HQ Cool Snap camera (Photometrics) , a 20× objective, a BP 470/20 nm exciter and a BP 505/530 nm emission filter. Intensity of fluorescence is used as a measure of prevalence of ROS. Data for each sample were collected from15 fields/treatment/sample. Image J software was employed for image analysis to obtain the fluorescence values. We acquired 20 cells with predefined features to calculate the average intensity.

### *Boolean Network Model of ROS Signalling*

Figure I shows a Boolean network model of ROS signalling in a targeted set of key genes participating in free radical metabolism. Although the signaling pathways to construct this model were primarily derived from published work on hepatoblastoma cell lines, cell line model of choice for ROS work, the fact that these genes are functionally conserved would make the network also suitable for studies involving other cancer cell lines including cell lines from breast cancer. A brief description of the network and the pathways involved follows.

Figure I (a) shows how increased electrophiles lead to disassociation of complex Keap1-Nrf2 (constituted by transcription factor Nrf2 and sensor Keap1). Released Nrf2 is then free to be transported to the nucleus. Figure I (b) illustrates Keap1 role, which is to sequester Nrf2 in the cytoplasm and also to enable the proteasomal degradation of Nrf2 when needed. Once dissociated from Keap1, Nrf2 is phosphorylated and transported to the nucleus. Inside the nucleus, Nrf2 forms heterodimers with small Maf proteins (SMP) which then binds to the antioxidant response element (ARE) and leads to the translation of genes of Phase II detoxifying enzymes and several other antioxidant proteins including chaperones, scavenger receptors, transporter proteins, proteasome proteins and transcription regulators. Also, PKC is known to be up-regulated along with Nrf2 under cellular conditions of oxidative stress. Furthermore Keap1 and Bach1 are known to be negative regulators of Nrf2 to complete this gene circuit. Once the electrophiles have been neutralized Nrf2 deactivation is carried out by other proteins to stop translation of the antioxidant genes. For instance, the Bach1-SMP (small Maf Proteins) complex, as well as small Maf hetero and homo dimer proteins are known to compete for the same region on the ARE as the Nrf2-SMP complex. So, once homeostasis of the cellular electrophile has been achieved, these protein complexes bind to the ARE and displace Nrf2 which is then transported back to the cytoplasm. In the cytoplasm, it binds with Keap1, which directs its proteosomal degradation. Figure I (c) shows a Boolean network of main genes consistent with their role in this pathway in response to elevated concentrations of electrophiles, various oxidant and antioxidant systems [10].

## Results

### *Results from Gene Expression Measurements Post Adriamycin and Cyclophosphamide Chemo-Agents Exposure at 4 and 24 Hours*

Results from this work reveal a complex pattern of directional and coordinated expression of functional gene groups in the tumorigenic cell lines both at 4 hours and 24 hours the two time points tested from the list of genes studied as predicted by our Boolean model (Figure I). This behavior was observed primarily in cancerous cell lines rather than in non-cancerous cell lines, partially validating the model tested. The gene expression results from this work did not validate the complete model and several reasons could be ascribed for this. One possibility is that in the construction of the original Boolean model all the changes were assumed to be synchronous and instantaneous which we know is not the case in a typical biological response. Second this study did not include all the known genes that are participants in this pathway nor all members of a given stress pathway including oxidative stress pathway that have yet to be fully characterized, knowledge that would be needed for developing more accurate models. Finally information from variables such as enzyme kinetics, biological events such as translocation of proteins from the nucleus to the cytoplasm or vice versa and protein modifications such as phosphorylation, acetylation and others may involve different time scales. The need for this information in turn mandates that future

theoretical to biological model validation studies should include several time points and parameters to confirm the model. We recognize that apart from the genes studied Figure I and II) there are several other genes that participate in this signaling pathway. However, for the purposes of this work we are mainly interested in the input (stress via addition of chemotherapeutic agents) -output (measurement of free radical generation and pattern of gene expression of the selected genes) relationships. From published literature, we have prior information on gene expression regulation pattern (up or down) of the genes studied here associated with oxidative-reductive changes in the cell milieu. This enables us to group the different genes based on their functional response. We anticipate two parameters to be fulfilled based on this information to validate our model. 1) Based on our current knowledge in response to oxidative stress we expect, ROS and the antioxidant genes, Nrf2 and PKC to be up-regulated whereas Bach1 and Keap1 will be down regulated. 2) Also, based on the Boolean model we predict that the direction of regulation of {Keap1, Bach1} will be in the opposite direction of {Nrf2, PKC}. At 24 hours (Fig II) for precancerous cell lines 10A and NeoT, we found statistically significant evidence of {Keap1, Bach1} showing similar direction of regulation that fits prediction from Boolean model whereas {Nrf2, PKC} showed opposite direction of regulation which does not fit the predicted model. In the case of tumorigenic cell lines 1h and 1a we found same direction of regulation for both pairs at 24 hours which fits the model. We however were not able to confirm in any given cell lines where both the above parameters i.e. {Nrf2, PKC} show same direction and {Keap1, Bach1} have same direction but opposite to that of {Nrf2, PKC} were satisfied. No statistically significant results were obtained for cyclophosphamide treatment at 24 hours, agreeing with early temporal activity of Cyclophosphamide [17]. Also at 4 hours there were no statistically significant results of any gene suggesting a dynamic flux of the system at that time point [18]. Our results support the notion that Boolean models can be applied for studying biological systems when requirements in experimental design are tightly met. For example in our case, by designing similar cell line based experiments with more time points we will be able to better capture the dynamic gene expression pattern and distinctly test our predicted Boolean model of oxidative stress. The cell lines are designated within the graph below the 0 line. Error bars indicate standard error. SOD3 although included in this study (as a negative control for gene expression) is expressed only in extracellular matrix and as expected was not expressed in the cell lines studied and not shown in this diagram. Also, at 4 hours none of the genes studied showed any discernable pattern and there is a much higher response of several phase II enzymes tested as quantified by real time PCR including SOD2 and HMOX1.

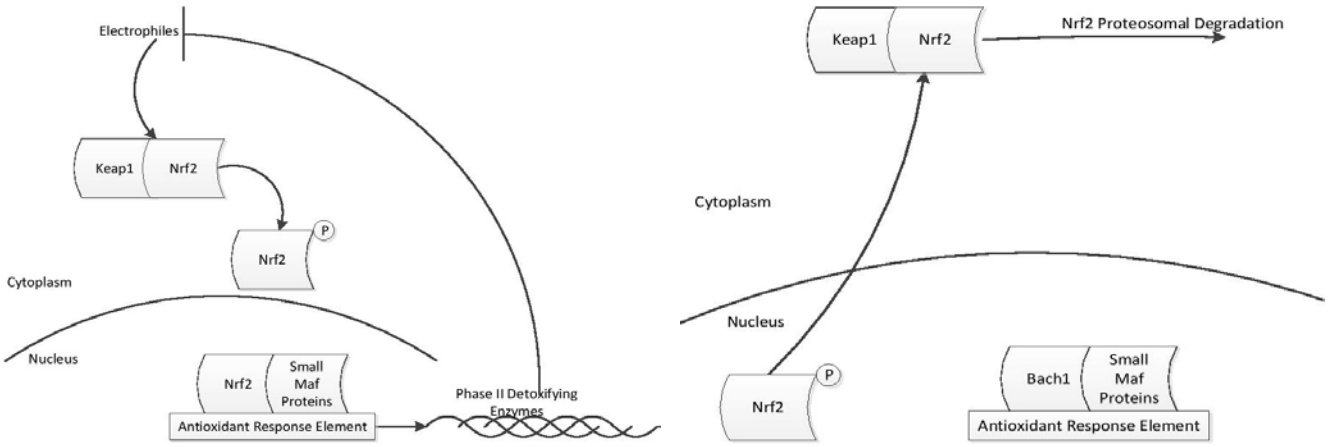
### *Results from Free Radical Measurement Post Adriamycin and Cyclophosphamidechemo-Agents Exposure at 4 and 24 Hours*

To determine the dynamics of free radical generation and its relationship to disease progression and chemotherapeutic agents we measured free radical generation at 4 and 24 hours. Our result was paradoxical in that cyclophosphamide (C) an alkylating chemotherapeutic agent that is known to generate less free radical in the therapeutic dose than Adriamycin (A) an anthracycline antibiotic [19], [20] actually produced more free radicals. At 24 hours 90% cases (9 out of 10, except NeoT treated with A+C) show increased free radical generation when C or A+C is applied when compared to A treatment. Similarly, even though there were no recognizable statistically significant patterns observed in 9 out 12 cases (75%)

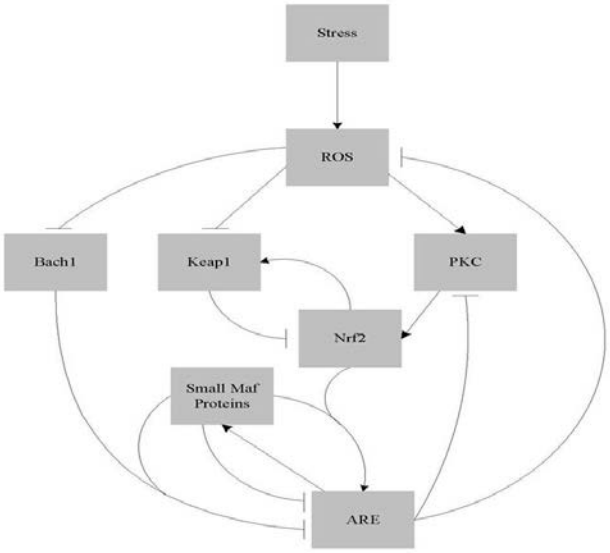
there was quantitatively more free radical generation at 24 hour time point as when compared to 4 hrs. We have proposed a model (Fig III) that may explain this unexpected behavior. Since A is known to produce more free radicals; it generates a free radical quenching response that is nonlinear and robust than in cases with cyclophosphamide treatments. Thus ROS generated at 24 hours post A treatment are seemingly less than ROS generated at the same time point post C treatment. This model can be validated with time course experiments. Finally, only in the case of the two cell lines genetically furthest removed from each other 10A (near normal) and 1a (most aggressive) showed a statistically significant difference up regulated in 3 cases and down regulated in 2 cases between 4 and 24 hours.

### *Statistical Analysis*

One tailed Students t-test was used to identify statistical significance 4 hours and 24 hours gene expression and free radical changes.




(a) NRF2 LOCALIZATION TO NUCLEUS (b) ELECTROPHILE NEUTRALIZATION




(c) BOOLEAN MODEL

FIGURE 1. THE BOOLEAN MODEL OF FREE RADICAL QUENCHING MECHANISM

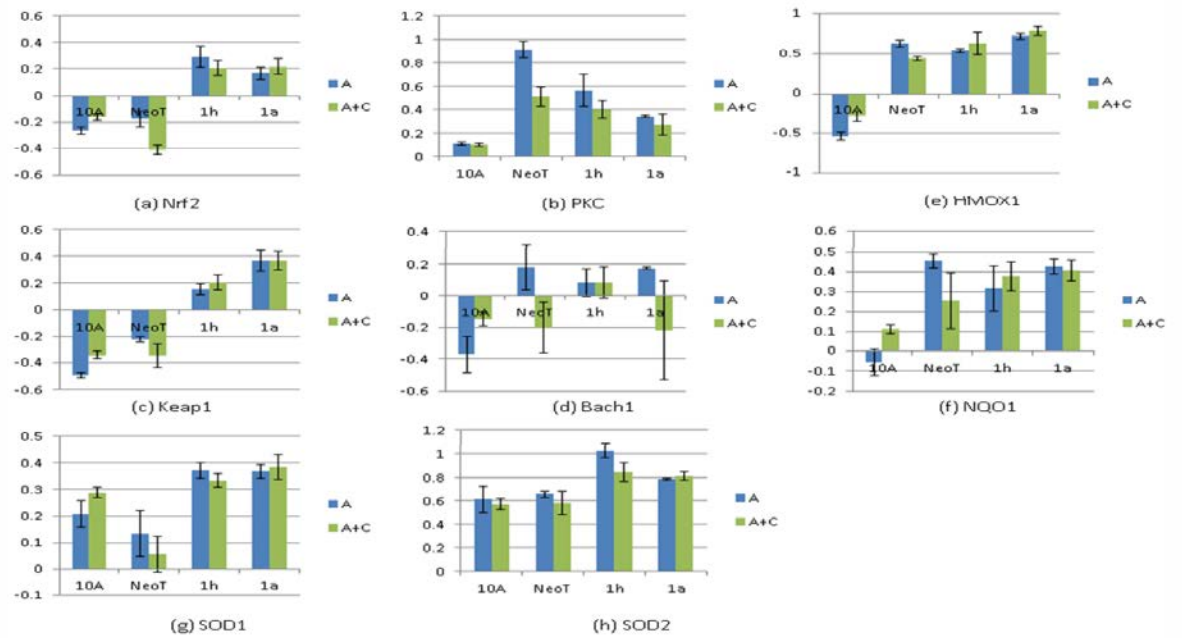



FIGURE 2. REGULATION OF ANTIOXIDANT RESPONSE GENES (ARE): (HMOX1, NQO1, SOD1 AND SOD2); STUDIED AT 24 HOURS THE COLOR LEGEND SPECIFIES THE TREATMENT

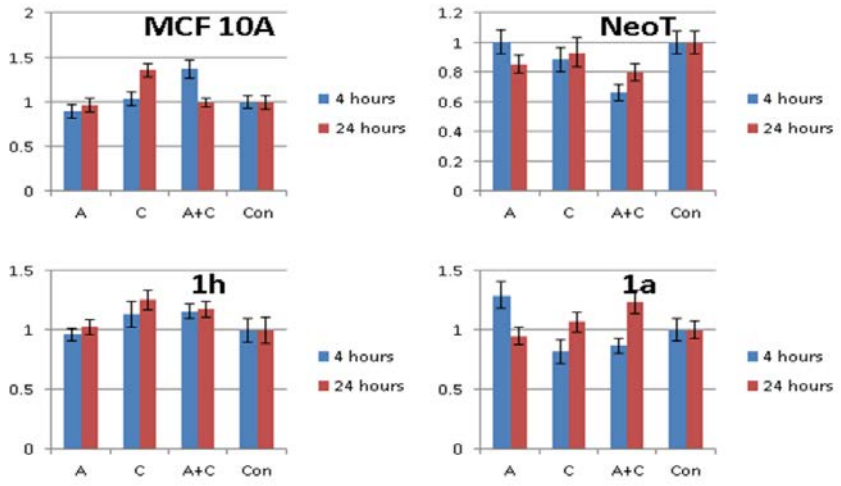



FIGURE 3. REGULATION OF FREE RADICALS

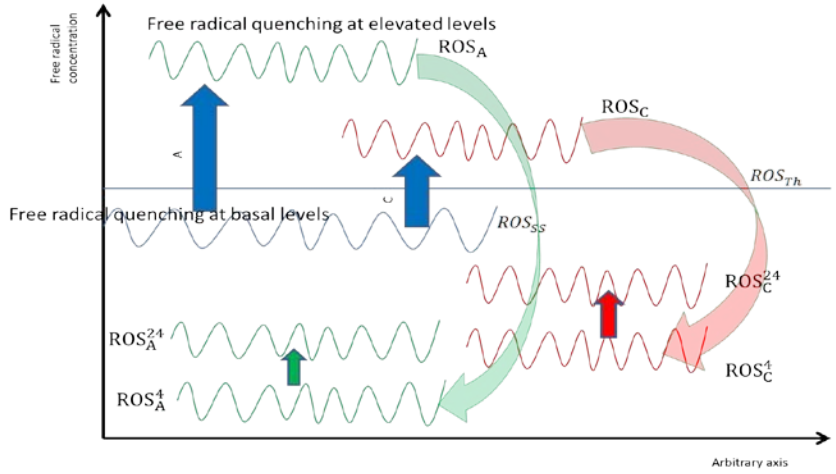



FIGURE 4. A PICTORIAL REPRESENTATION OF THE MODEL BASED ON FREE RADICAL MEASUREMENT

## Discussion

The main aim of this study was to shed light on the dynamics of reactive oxygen species (ROS) generation and response by a group of genes mitigating this process in a progressive series of breast cancer cell lines after chemotherapy. The two commonly used chemo-agents Adriamycin and Cyclophosphamide were evaluated at two time intervals 4 hours and 24 hours post exposure to a therapeutic dose. Both the dose and time selected to evaluate gene expression response were crucial in this study. The two time points selected; 4 hours offers a window of activity within a time period where there is peak activity of the drugs and 24 hours a point wherein the next dose of drugs are administered to the patient. Also a therapeutic dose was chosen to reflect real-world parameters for this study. The progressive nature of the breast cancer cell line allows for exploring the quenching mechanism in early and late stages of breast cancer progression. We did not observe a pattern either in gene expression response or free radical generation that could categorize tumorigenic and non-tumorigenic cell lines into discrete sets. These findings are not entirely unexpected because ROS generation is tightly regulated through a vast number of inducers and inhibitors that are not a part of this study. However increased time course analyses may increase the possibility of identifying a pattern if it exists. Our experimental results highlight the fact that free radical quenching might not be as simple as predicted by the Boolean model. There might be other non-linear factors associated based on the "threshold" concept as illustrated by us in the results section and further explained below. In either situation when exposed to ROS the cell reacts through up regulating quenching mechanisms to maintain redox homeostasis.

ROS arise as products of endogenous energy metabolism and also from exogenous sources, like radiation, air

pollutants and tobacco smoke, or through the metabolism of certain drugs, including antineoplastic agents such as doxorubicin, cyclophosphamide, pesticides, and solvents. Once produced they can react to molecules within the cell and in the extra cellular space. To counteract ROS induced oxidative stress, aerobic organisms have developed antioxidant defenses, including enzymes (superoxide dismutase, catalase, NQO1), beside small molecular weight endogenous antioxidants which include reduced glutathione (GSH) , total thiols (T-SH), as well as certain amino acids and vitamins. There is a delicate equilibrium that is maintained between generation and quenching of free radicals, known as oxidative balance [21]. There is a threshold beyond which the balance between free radical generation and quenching is tilted towards free radical generation leading to insufficient quenching. A slight imbalance by oxidative stress can be reversed to the original state of cells through the cellular auto regulation mechanism. Nevertheless, more severe oxidative stress after crossing certain threshold (based on a cell microenvironment) has destructive effects on cells, leading to cell dysfunction or cell death [22]. In addition, excess free radicals react with several cellular substrates including the nucleotides in the DNA resulting in mutations that are consequential in initiation and progression of cancer. Based on our results as depicted in Fig II, III and the model proposed by Fig IV we hypothesize chemo-agents such as Adriamycin and Cyclophosphamide which are well known generators of ROS as the ROS level cross a threshold activate a non-linear free radical quenching beyond the proportional response. Earlier work has shown this phenomenon of nonlinearity in ROS generation based on chemicals and concentration used and the oxidative status of the cell [23]. Based on a PC12 model of a rat pheochromocytoma cell line, there was linear variation of fluorescence with increasing concentrations (between 0.1 and 1 mM) of H2O2 with the compound 2,29-azobios(2-amidinopropane) dihydrochloride (AAPH; a peroxyl radical generator). By contrast, the fluorescence varied with nonlinear response to increasing concentrations of compounds 3 morpholinosydnonimine hydrochloride (SIN-1; a peroxynitrite generator), sodium nitroprusside (SNP; a nitric oxide generator), and dopamine. Moreover, dopamine had a biphasic effect; it decreased the DCF fluorescence, thus acting as an antioxidant, at concentrations , 500 mM in cells, but acted as a pro-oxidant by increasing the fluorescence at 1 mM. In context of cancer cell milieu, low levels of ROS generation have been proposed to lead to cell proliferation, whereas high levels to apoptosis and cell death. Also there is good experimental corroboration that cancer cells may be more capable in adapting to ROS and thus are more resistant to ROS as when compared to normal cells. Our results point to the possibility of cancer cells exhibiting threshold effects in the level of response to ROS generation which may also play a part in its therapeutic efficacy. Moreover, results from this work suggest that free radical generation is nonlinear and concentration based, initially correlated to the concentration of the chemotherapy agents, but after a certain time due to nonlinear cellular quenching response, free radical generation and availability in the cell milieu is reduced significantly, in turn reducing the potency of the drug's tumor killing ability [23]. Taken together this may explain some of controversy about the possible limitations in drug effectiveness caused by an excess of free radicals [3], [24]. The traditional "maximum tolerated dose" has been accompanied with overt toxicity demanding rest periods between cycles of therapy. This practice has resulted in re-growth of tumor cells and selection of clones resistant to chemotherapy [25], [26], [27], [28], [29]. To avoid this pitfall in conventional chemotherapeutic regimens, a new modality of drug administration called "metronomic chemotherapy" has been suggested [30]. The practice of metronomic chemotherapy which entails chronic low dose chemotherapy exposure has proven to be a valuable tool in the fight against cancer and has quickly moved from clinical trials to a mainstream therapeutic option. To date several articles have been published using various chemotherapeutic agents for several types of cancer with encouraging results in both pediatric and adult settings [25], [26], [27], [28]. Furthermore, our results support the idea of administering low doses in equally spaced administration cycles, over the traditional high dose protocols with imposition of rest periods due to overt toxicity. This low dose continuous treatment is a better approach in order to both reduce toxicity and achieve overall better tumor killing and clinical success. This paradigm of using low-dose therapy to alter the tumor microenvironment to alter cancer dynamics has been suggested to be more effective than the traditional method [35]. There is a constant effort to balance between efficacy in tumor killing and toxicity of the chemotherapeutic agents. To the best of our knowledge, our data may provide preliminary evidence the advantage of metronomic chemotherapy from the standpoint of ROS generation and quenching mechanism during chemotherapy. Moreover, our results point to the importance of chemotherapeutic regimens in determining the efficacy of cancer treatment. The hypothesis generated from this work can be validated by designing experiments that take into consideration the dosage and timing of drug applications in combination with measurements of ROS

generation, cell proliferation and apoptosis. In our previous work [10], we modelled and simulated free radical generation quenching cycles applying the principle of oscillation in biological networks. As expected, the results exhibited oscillatory behavior in the presence of free radicals. By oscillatory behavior the proposition is that in the presence of continuous free radicals stimulant (chemo-agents Adriamycin and Cyclophosphamide), there is a corresponding gene transcription oscillated between the maximal and minimal parameters. The initial influx of stimulant causes production of free radicals, to which the genes that participate in the anti-oxidant network respond trying to quench it. This drives free radical break down towards basal levels. But because there is a continuous rate of influx of stimulants, there is a pro-oxidant intercellular milieu with time and the accompanying quenching response. This oscillatory behavior follows until the stimulants for pro-oxidants are removed. Our results based on a qualitative biological measurement of behavior validated this premise in a majority, though not all, of the instances as explained in the results section. The Boolean model considers the presence of its components as a switch but does not take into account the exact quantitative levels in this context [10]. An important point to be considered as we bridge mathematical models with biological validation experiments is the fact that most current mathematical models are simple models not taking into account layers of complexity in the biological systems including feedback loops that control in many instances the on and off switches [36], [37],[10] . As we increase the generation of data wherein theoretical models and biological wet laboratory validation are done in parallel, data derived from wet laboratory work will reinforce and often times correct the model. The availability of global gene analysis tools such as micro and macro arrays to analyze thousands of gene products in a single hybridization at different time points and approaches with automated technology such as digital PCR and microfluidics will support these types of approaches. In particular the microfluidics based digital PCR is a very sensitive technology that has the potential to accurately measure to quantify nucleic acid in a very small sample through counting individual DNA molecules and this will prove to be an effective tool for the type of methodology that needs large data generated from several time points [38], [39], [40].

## Conclusions

In conclusion, we have shown that experimental validation of “multivariate digital modelling” of gene regulation through building Boolean Networks may be feasible. But as in any early amalgamations of technology, our current attempt to validate a mathematical logic based Boolean circuit network through a real time PCR based gene expression study needs constant parallel fine tuning on both ends to bridge the gaps in information. This will in turn build considerable knowledge to make it a robust approach in time. We have also provided for the preliminary data through a ROS mediated effect to support “metronomic chemotherapy” which has proven to show a real promising future in the current battle against cancer. It is conceivable that the accumulated knowledge acquired through this and other related work will spark a change in the way therapeutic protocols are designed in treating cancer.

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